

Claims

1. A method for increasing the number of copies of an amplification unit integrated into a host cell chromosome, wherein the method comprises the steps of:

5 a) rendering a chromosomal gene of a host cell non-functional, wherein the host cell becomes susceptible to an inhibitory compound endogenously produced by the host cell when the host cell is cultivated in a medium comprising a precursor;

b) making a nucleic acid construct comprising an amplification unit, wherein the unit comprises:

10 i) an expression cassette comprising at least one copy of a gene of interest; and

ii) an expressable copy of the chromosomal gene of step a), wherein the unit integrates into the host cell chromosome;

c) introducing the nucleic acid construct of step b) into the host cell of step a), wherein at least one copy of the amplification unit integrates into the host cell chromosome;

5 d) cultivating the host cell of step c) in a medium comprising the precursor, wherein a chromosomally integrated copy of the amplification unit is duplicated or multiplied on the host cell chromosome;

e) selecting a host cell comprising two or more chromosomally integrated copies of the amplification unit; and optionally

20 f) performing one or more cycles of steps d) and e) using the host cell selected in step e) in each new cycle; wherein the number of chromosomally integrated copies of the amplification unit increases with each repeat.

2. A method for constructing a host cell comprising at least one copy of an amplification unit integrated into the host cell chromosome, wherein the method comprises the steps of:

25 a) rendering a chromosomal gene of a host cell non-functional, wherein the host cell becomes susceptible to an inhibitory compound endogenously produced by the host cell when the host cell is cultivated in a medium comprising a precursor;

30 b) making a nucleic acid construct comprising an amplification unit, wherein the unit comprises:

i) an expression cassette comprising at least one copy of a gene of interest; and

ii) an expressable copy of the chromosomal gene of step a), wherein the unit integrates into the host cell chromosome;

c) introducing the nucleic acid construct of step b) into the host cell of step a) and cultivating the host cell in a medium comprising the precursor, wherein at least one copy of the amplification unit integrates into the host cell chromosome; and

5 d) selecting a host cell comprising at least one chromosomally integrated copy of the amplification unit.

3. A method for increasing the number of copies of an amplification unit integrated into a host cell chromosome, wherein the method comprises the steps of:

10 a) providing a host cell, wherein a chromosomal gene has been rendered non-functional, whereby the host cell becomes susceptible to an inhibitory compound endogenously produced by the host cell when the host cell is cultivated in a medium comprising a precursor;

b) introducing a nucleic acid construct into the host cell of step a), the nucleic acid construct comprising an amplification unit, wherein the unit comprises:

5 i) an expression cassette comprising at least one copy of a gene of interest; and

ii) an expressible copy of the chromosomal gene of step a),

wherein at least one copy of the amplification unit integrates into the host cell chromosome;

c) cultivating the host cell of step b) in a medium comprising the precursor, wherein a chromosomally integrated copy of the amplification unit is duplicated or multiplied on the host cell chromosome;

0 d) selecting a host cell comprising two or more chromosomally integrated copies of the amplification unit; and optionally

e) performing one or more cycles of steps c) and d) using the host cell selected in step d) in each new cycle; wherein the number of chromosomally integrated copies of the amplification unit increases with each cycle.

25

4. The method of claim 1, wherein the host cell is a *Bacillus* cell selected from the group consisting of *Bacillus alkalophilus*, *Bacillus amyloliquefaciens*, *Bacillus brevis*, *Bacillus circulans*, *Bacillus clausii*, *Bacillus coagulans*, *Bacillus lautus*, *Bacillus lentus*, *Bacillus licheniformis*, *Bacillus megaterium*, *Bacillus stearothermophilus*, *Bacillus subtilis*, and *Bacillus thuringiensis*.

30

5. The method of claim 1, wherein the chromosomal gene of step a) encodes an enzyme selected from the group consisting of galactokinase (EC 2.7.1.6), UTP-dependent

pyrophosphorylase (EC 2.7.7.10), UDP-glucose-dependent uridylyltransferase (EC 2.7.7.12), and UDP-galactose epimerase (EC 5.1.2.3).

6. The method of claim 1, wherein the chromosomal gene of step a) encodes an enzyme
5 with UDP-galactose epimerase activity (EC 5.1.2.3).

7. The method of claim 1, wherein the chromosomal gene of step a) is galE.

8. The method of claim 1, wherein the inhibitory compound is UDP-galactose.

10 9. The method of claim 1, wherein the precursor is free galactose.

10. The method of claim 1, wherein the precursor can be degraded to produce free
5 galactose.

11. The method of claim 1, wherein the precursor is lactose, melibiose, raffinose, stachyose,
verbascose or galactinol.

12. The method of claim 1, wherein the medium comprises an enzyme capable of degrading
10 the precursor to produce free galactose.

13. The method of claim 1, wherein the host cell secretes an enzyme into the medium which
is capable of degrading the precursor to produce free galactose.

25 14. The method of claim 12, wherein the enzyme is a galactosidase.

15. The method of claim 1, wherein the nucleic acid construct is a plasmid.

16. The method of claim 1, wherein the nucleic acid construct further comprises an antibiotic
30 selection marker flanked by resolvase sites or res-sites.

17. The method of claim 1, wherein the amplification unit further comprises a nucleotide
sequence with a homology to a chromosomal nucleotide sequence of the host cell sufficient to

effect chromosomal integration in the host cell of the amplification unit by homologous recombination.

18. The method of claim 1, wherein the amplification unit further comprises a nucleotide
5 sequence of at least 100 bp, preferably 200 bp, more preferably 300 bp, even more preferably
400 bp, and most preferably at least 500 bp with an identity of at least 70%, preferably 80%,
more preferably 90%, even more preferably 95%, and most preferably at least 98% identity to a
chromosomal nucleotide sequence of the host cell.

10 19. The method of claim 17, wherein the nucleotide sequence comprised in the amplification
unit is a partial non-functional copy of a conditionally essential gene of the host cell, wherein the
host cell prior to the first step of the invention has had the conditionally essential gene rendered
non functional by a partial deletion, and wherein a recombination event between the partial
copy of the gene comprised in the amplification unit and the partial chromosomal gene restores
5 a functional chromosomal gene.

20. The method of claim 19, wherein the conditionally essential gene encodes a D-alanine
racemase.

20 21. The method of claim 19, wherein a first amplification unit integrates into the host cell
chromosome by homologous recombination with the partially deleted conditionally essential
gene and renders the gene functional.

22. The method of claim 1, wherein the amplification unit further comprises an antibiotic
25 marker, preferably flanked by resolvase sites or res-sites.

23. The method of claim 22, wherein a host cell comprising a first chromosomally integrated
amplification unit is selected and the antibiotic marker excised from the host cell chromosome
by a resolvase prior to the next step in the method.

30 24. The method of claim 1, wherein the gene of interest encodes a polypeptide of interest.

25. The method of claim 24, wherein the polypeptide is an enzyme such as a protease; a cellulase; a lipase; a xylanase; a phospholipase; or preferably an amylase.

26. The method of claim 24, wherein the polypeptide is a hormone, a pro-hormone, a pre-
5 pro-hormone, a small peptide, a receptor, or a neuropeptide.

27. The method of claim 1, wherein the expressable copy of the chromosomal gene comprised in an amplification unit integrated in the host cell chromosome has a reduced transcription level compared to the transcription level of the wild type gene of the host cell,
10 preferably the transcription level is reduced with a factor of 100, preferably 50, more preferably 10, even more preferably 5, and most preferably with a factor of 2.

28. The method of claim 1, wherein the expressable copy of the chromosomal gene comprised in the amplification unit is promoterless.

29. The method of claim 1, wherein the expressable copy of the chromosomal gene comprised in the amplification unit has a transcription terminator located upstream of the gene.

30. The method of claim 1, wherein the gene of interest is located upstream of the expressable copy of the chromosomal gene within the amplification unit and wherein the two
0 genes are co-directionally transcribed.

31. The method of claim 30, wherein the expressable copy of the chromosomal gene is expressed by read-through transcription from the gene of interest.

25 32. An amplification unit comprising:
a) an expression cassette comprising at least one copy of a gene of interest; and
b) an expressable copy of a conditionally essential chromosomal gene of a host cell;
wherein the unit integrates into the host cell chromosome upon introduction of the nucleic acid
30 construct into the host cell.

33. The unit of claim 32, wherein the chromosomal gene encodes an enzyme, preferably chosen from the group consisting of galactokinase (EC 2.7.1.6), UTP-dependent

pyrophosphorylase (EC 2.7.7.10), UDP-glucose-dependent uridylyltransferase (EC 2.7.7.12), UDP-galactose epimerase (EC 5.1.2.3).

34. The unit of claim 32, wherein the chromosomal gene encodes an enzyme with UDP-
5 galactose epimerase activity (EC 5.1.2.3).

35. The unit of claim 32, wherein the chromosomal gene is galE.

36. The unit of claim 32, wherein the gene of interest encodes a polypeptide of interest.

10 37. The unit of claim 36, wherein the polypeptide is an enzyme such as a protease; a cellulase; a lipase; a xylanase; a phospholipase; or preferably an amylase.

15 38. The unit of claim 36, wherein the polypeptide is a hormone, a pro-hormone, a pre-pro-hormone, a small peptide, a receptor, or a neuropeptide.

39. The unit of claim 32, wherein the expressable copy of the chromosomal gene is promoterless.

20 40. The unit of claim 32, wherein the expressable copy of the chromosomal gene has a transcription terminator located upstream of the gene.

41. The unit of claim 32, wherein the gene of interest is located upstream of the expressable copy of the chromosomal gene and wherein the two genes are co-directionally transcribed.

25 42. The unit of claim 41, wherein the expressable copy of the chromosomal gene is expressed by read-through transcription from the gene of interest.

30 43. The unit of claim 32, which further comprises an antibiotic marker, preferably flanked by resolvase sites or res-sites.

44. A nucleic acid construct comprising a unit of claim 32.

45. A host cell wherein a chromosomal gene has been rendered non-functional leaving the host cell susceptible to an inhibitory compound endogenously produced by the host cell when cultivated in a medium comprising a precursor; and wherein the host cell comprises an amplification unit of claim 32.

5

46. The host cell of claim 45, wherein the host cell is a Gram-positive bacterial cell, preferably a *Bacillus* cell, more preferably a *Bacillus* cell of a species chosen from the group consisting of *Bacillus alkalophilus*, *Bacillus amyloliquefaciens*, *Bacillus brevis*, *Bacillus circulans*, *Bacillus clausii*, *Bacillus coagulans*, *Bacillus lautus*, *Bacillus lentus*, *Bacillus licheniformis*,
10 *Bacillus megaterium*, *Bacillus stearothermophilus*, *Bacillus subtilis*, and *Bacillus thuringiensis*; and most preferably a *Bacillus licheniformis* cell.

5

47. The host cell of claim 45, wherein the chromosomal gene encodes an enzyme, preferably the enzyme is chosen from the group of enzymes consisting of galactokinase (EC 2.7.1.6), UTP-dependent pyrophosphorylase (EC 2.7.7.10), UDP-glucose-dependent uridylyltransferase (EC 2.7.7.12), UDP-galactose epimerase (EC 5.1.2.3), more preferably the enzyme is an UDP-galactose epimerase (EC 5.1.2.3), and most preferably the enzyme is encoded by galE.

0

48. The host cell of claim 45, where the inhibitory compound is UDP-galactose.

49. The host cell of claim 45, where the precursor is free galactose, preferably free D-galactose.

25 50. The host cell of claim 45, where the precursor can be degraded to produce free galactose, or preferably free D-galactose.

51. The host cell of claim 45, where the precursor is lactose, melibiose, raffinose, stachyose, verbascose or galactinol.

30

52. The host cell of claim 45, where the medium comprises an enzyme capable of degrading the precursor to produce free galactose, or preferably free D-galactose.

53. The host cell of claim 45, where the host cell secretes an enzyme into the medium which is capable of degrading the precursor to produce free galactose, or preferably free D-galactose.

5 54. The host cell of claim 52, where the enzyme is a galactosidase, preferably an alpha-galactosidase or a beta-galactosidase.

55. The host cell of claim 45, wherein the amplification unit further comprises a nucleotide sequence of at least 100 bp, preferably 200 bp, more preferably 300 bp, even more preferably
10 400 bp, and most preferably at least 500 bp with an identity of at least 70%, preferably 80%, more preferably 90%, even more preferably 95%, and most preferably at least 98% identity to a chromosomal nucleotide sequence of the host cell.

56. The host cell of claim 55 wherein the nucleotide sequence comprised in the amplification
5 unit is a partial non-functional copy of a conditionally essential gene of the host cell, wherein the host cell has had the conditionally essential gene rendered non functional by a partial deletion, and wherein a recombination event between the partial copy of the gene comprised in the amplification unit and the partial chromosomal gene has restored a functional chromosomal gene.

57. The host cell of claim 56 wherein the conditionally essential gene encodes a D-alanine racemase, preferably the conditionally essential gene is dal.

58. The host cell of claim 45 wherein the expressable copy of the chromosomal gene of the
25 amplification unit has a reduced transcription level compared to the transcription level of the wild type gene of the host cell, preferably the transcription level is reduced with a factor of 100, preferably 50, more preferably 10, even more preferably 5, and most preferably with a factor of 2.

30 59. A process for producing a polypeptide of interest, wherein the process comprises a step of cultivating a host cell of claim 45.

60. The process of claim 59 wherein the polypeptide is an enzyme such as a protease; a cellulase; a lipase; a xylanase; a phospholipase; or preferably an amylase.

61. The process of claim 59 wherein the polypeptide is a hormone, a pro-hormone, a pre-
5 pro-hormone, a small peptide, a receptor, or a neuropeptide.